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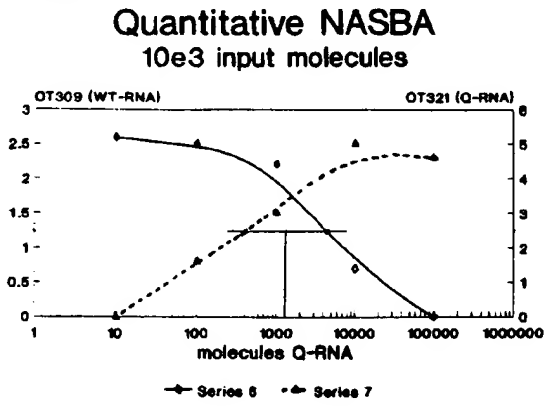
(54) **Quantification of nucleic acid.**

(57) Disclosed is a method of quantifying a target nucleic acid in a test sample by adding to the test sample a known number of molecules of a corresponding nucleic acid comprising a well-defined mutant sequence. Said mutant sequence being discriminatory from the target nucleic acid. Subsequently a competitive amplification reaction of the nucleic acid is performed after which quantification of the amplified nucleic acid is performed by a differential detection.

FIGURE 1



EP 0 525 882 A1

The invention relates to a method for quantification of target nucleic acid in a test sample. A test kit for carrying out said method is also part of the invention.

A method for carrying out the amplification of nucleic acid in a test sample has been disclosed among others by Cetus Corp. in USP 4,683,195 and 4,683,202 the so-called polymerase chain reaction (PCR).

Recently another method for amplification of nucleic acid in a test sample, especially RNA sequences, has been disclosed in European Patent Application EP 0,329,822 by Cangene Corp. The process itself will not be discussed here in detail, but it concerns the so-called NASBA™ technique (= nucleic acid sequence based amplification).

Amplification is an exponential process. Small differences in any of the variables which control the reaction rate will lead to dramatic differences in the yield of the amplified product. PCR as well as NASBA have wide-spread applications in genetic disease diagnosis however, these techniques only provide qualitative results.

A need exists for a method of quantifying directly, accurately, and in a reproducible manner, the amount of a specific nucleic acid present in a test sample.

A sensitive, reproducible, quantitative analysis of a test sample obtained from a patient suffering from an infectious disease, e.g. AIDS or hepatitis, can be of utmost importance in determining the extent of the infectious agent present in the patient, which information is useful in monitoring the patient treatment.

The present invention provides a method of quantifying a target nucleic acid in a test sample comprising adding a known number of molecules of a corresponding nucleic acid comprising a well-defined mutant sequence to the test sample, said mutant sequence being discriminatory from the target nucleic acid, but amplifiable with comparable efficiency, subsequently performing an amplification reaction of the nucleic acid, after which quantification of the amplified nucleic acid is performed by differential detection.

The target nucleic acid can be deoxyribonucleic acid (DNA) as well as ribonucleic acid (RNA).

Preferably the target nucleic acid sequence is ribonucleic acid. The differential detection necessary in this method is performed by using a probe sequence able to hybridize with both the target nucleic acid and the mutant sequence as well, or using two probes discriminating the target sequence and mutant sequence.

Said differentiation can also be performed by using a ribozyme capable of cleaving the mutant sequence, while the target sequence will not be cleaved by the ribozyme used or vice versa.

A part of the invention includes a test kit for carrying out the previously described methods.

Recently patent application WO 91/02817 was published in which a co-amplification of an internal standard nucleic acid segment and target sequence was described. The method used in this application is not a competition reaction. In contrast to the instant invention quantification in that application is performed by measuring the signals obtained and subsequently determining the ratio between both sequences amplified. The present invention differs significantly from that process since, among other things, competition between wild-type (target nucleic acid) and well-defined mutant sequence is an essential part of the instant invention.

The method according to the instant invention is based on the principle of competitive amplification of nucleic acid from a clinical sample containing an unknown concentration of wild-type target nucleic acid, to which has been added a known amount of a well-defined mutant sequence.

Amplification of both target nucleic acid and mutant sequence as well is preferably performed with one primer set including two primers of which each primer hybridizes to the target nucleic acid and mutant sequence with the same efficiency.

This competitive amplification is performed with a fixed amount of (clinical) sample and dilution series of mutant sequence or vice versa.

The mutation in the added sequence is necessary for discriminatory detection of the wild-type and mutated amplified sequences with wild-type and mutation specific labelled oligonucleotides respectively.

This means that after competitive amplification samples are analysed in duplo using any sequence specific detection, for example:

1. gelelectrophoresis, blotting, hybridization, autoradiography, scanning;
2. Slot-blotting, hybridization, autoradiography, scanning.;
3. Non-capture bead based assay, counting; and
4. Capture bead based assay, counting.

The initial ratio of wild-type and mutated sequence will be found back in the ratio of wild-type and mutated signals. At a 1:1 ratio and equal efficiency of amplification, the reduction in signal for both wild-type and mutated sequence will be 50%. So at the dilution of mutated nucleic acid that causes a 50% reduction in signal the amount of mutated nucleic acid equals the amount of wild-type nucleic acid in the (clinical) sample.

Using a well-defined mutant sequence comprising, for instance, in the sequence a single base mutation

(e.g. an A -> G transition) just one restriction enzyme, or a ribozyme, has to be used to discriminate between target nucleic acid and the mutant sequence.

Subsequently just one analysis running (for instance one gel system) is necessary in order to quantify the target nucleic acid.

Samples suitable for analysis by this method may be of human or non-human origin. The samples may be derived from cultured samples, for instance, mononuclear cells, or isolated from dissected tissue. Also blood and blood plasma, as well as brain-liquor, urine, etc. can be used as test sample material.

If, for example the test sample is blood with a target virus to be quantified according to the invention, the viral nucleic acid can be extracted from the test sample. In order to obtain a very fast, simple and reproducible procedure according to the invention the well-defined mutant sequence can be added before, during or after the target nucleic acid extraction without interference in the extraction procedure. Subsequently the competitive amplification and differential detection according to the invention can be performed directly after the extraction procedure.

Due to its high sensitivity, speed, reproducibility and accuracy, the present method can be used to quantify exactly the amount of, for instance, viruses like AIDS-virus or hepatitis virus in the test sample obtained from a patient suspected of suffering from the disease.

It can be of prime importance to know at different stages in a disease the exact amount of viruses or other disease-causing agents in order, for example, to know the dose of medication to be administered to the patient.

The test kit according to the invention is provided in its simplest embodiment with a well-defined mutant sequence and appropriate oligonucleotides viz. primers/primer pair in order to perform the desired amplification reaction and a probe sequence or ribozyme as well.

Additionally, a test kit can be supplied with the appropiate enzymes in order to carry out the amplification reaction.

The method according to the invention is illustrated by the following examples.

Example I

In vitro generated wild-type (WT) and mutant (Q) RNA were used to prove the principle of quantitative NASBA™. Plasmids used for in vitro RNA synthesis contained a 1416 bp fragment of the HIV-1 sequence resulting from a partial Fok 1 restriction enzyme digest (nucleotides 1186-2638 of the HIV-1hxb2 sequence, Ratner et al., 1987) cloned in pGEM3 or pGEM4 (Promega). The sequence between the restriction sites PstI (position 1418 on HIV-1 hxb2) and Sph I (position 1446 on HIV-1 hxb2) was changed from GAATGGGATAGAGTGCATCCAGTGCATG (OT309) in the WT to GACAGTGTAGATAGATGACAGTCGCATG (OT321) in the Q RNA. In vitro RNA was generated from these constructs with either T7 RNA polymerase or SP6 RNA polymerase. (Sambrook et al., 1989).

Reaction mixtures were treated with DNase to remove plasmid DNA. After phenol extraction and ethanol precipitation the recovered RNA was quantitated on ethidium bromide stained agarose gels by comparison to a calibration series of known amounts of ribosomal RNA. The RNA solutions were diluted to the desired concentrations and used as input for amplification by NASBA™ as described in EP 0329,822. Primers used for amplification were OT 270: (AATTCTAATACGACTCACTATAGGGGTGCTATGTCACTTCCCCTTGGTTCTCTCA , P1) and OT271 (AGTGGGGGGACATCAAGCAGCCATGCAAA, P2), generating a RNA molecule complementary to the HIV-1hxb2 sequence of 142 nt (pos 1357 to 1499). Detection of 10 µl of each amplification has been performed by electrophoresis in duplo on 3% NuSieve, 1% agarose gels (Sambrook et al., 1989) blotted onto Zeta-Probe (Biorad) using a vacuumblot apparatus (Pharmacia) and hybridized with ^{32}P labelled oligonucleotides specific for either the WT or the Q RNA sequence between above mentioned Sph1 and Pst 1 sites. Exposure times to X-ray films (Kodak) ranged from 30 minutes to 3 days.

Films were scanned with a LKB Ultroscan XL densitometer for quantification of the signal in the bands. Number of target molecules of both WT and Q RNA are listed in table 1.

Table 1

Tube	Copies W.T. RNA	Copies Q RNA
1	10^3	10^1
2	10^3	10^2
3	10^3	10^3
4	10^3	10^4
5	10^3	10^5

As control amplification of WT RNA or Q RNA alone was performed. The results of the competitive NASBA™ are presented in fig. 1. At the mean of the 50% reduction for both WT and Q RNA the number of input molecules is approximately 10^3 molecules Q RNA, which equals the number of WT RNA molecules.

The formula used for determining the mean of 50% reduction for both Q and WT RNA is as follows:

$$\log (\text{conc. W.T.}) = \frac{\log ([Q]\ 50\%\ \text{Sig Q}) + \log ([Q]\ 50\%\ \text{Sig. WT})}{2}$$

in which ([Q] 50% Sig. Q) is the number of Q RNA molecules at which the signal using OT 321, specific for Q RNA, is only 50% of the signal obtained when Q RNA alone is amplified and ([Q] 50% Sig. WT) is the number of Q RNA molecules at which the signal using OT 309, specific for WT RNA, is only 50% of the signal obtained when WT RNA alone is amplified.

Example II

As in example 1 except input RNA molecules are as in table 2.

Table 2

Tube	copies W.T. RNA	copies Q RNA
1	10^4	10^2
2	10^4	10^3
3	10^4	10^4
4	10^4	10^5
5	10^4	10^6

The results presented in fig. 2 show an input of 10^4 molecules of WT RNA using the formula.

$$\log. (\text{conc. WT}) = \frac{\log ([Q]\ \text{Sig Q}\ 50\%) + \log ([Q]\ \text{Sig. WT}\ 50\%)}{2}$$

Example III

As in example 1 except that input RNA molecules are as in table 3.

Table 3

Tube	copies W.T. RNA	copies Q RNA
1	10^5	10^3
2	10^5	10^4
3	10^5	10^5
4	10^5	10^6
5	10^5	10^7

The results presented in fig. 3 show an input of 6.5×10^4 molecules of WT RNA using the formula.

$$\log (\text{conc. W.T.}) = \frac{\log ([Q] \text{ Sig Q } 50\%) + \log ([Q] \text{ Sig. WT } 50\%)}{2}$$

Example IV

Here quantitative NASBA^{tm} is applied to nucleic acid isolated from plasma of HIV-1 infected individuals. 1 ml plasma samples of 3 sero-positive HIV-1 infected individuals were used to isolate nucleic acid (Boom et al., 1990).

Nucleic acid was finally recovered in 100 µl water. Amplifications were as in example 1 except input RNA molecules were as in table 4.

Table 4

tube	volume nucleic acid sol.	copies Q RNA
1	2 µl patient 1	10^1
2	2 µl patient 1	10^2
3	2 µl patient 1	10^3
4	2 µl patient 1	10^4
5	2 µl patient 1	10^5
6	2 µl patient 2	10^1
7	2 µl patient 2	10^2
8	2 µl patient 2	10^3
9	2 µl patient 2	10^4
10	2 µl patient 2	10^5
11	2 µl patient 3	10^1
12	2 µl patient 3	10^2
13	2 µl patient 3	10^3
14	2 µl patient 3	10^4
15	2 µl patient 3	10^5

Results are presented in figures 4, 5 and 6 for patients 1, 2 and 3, respectively.

Results indicate the number of W.T. RNA molecules for patients 1, 2 and 3 to be 4.5×10^3, 2.1×10^3 and 1.2×10^4 in 2µl nucleic acid solution, respectively, using the formula:

$$\log (\text{conc. WT}) = \frac{\log ([Q] \text{ Sig Q } 50\%) + \log ([Q] \text{ Sig. WT } 50\%)}{2}$$

Example V

As in example 1 except that input RNA molecules are as in table 5 and that detection of NASBA amplified WT^- and Q-RNA is according to the hereafter described method.

Amplified WT^- and Q-RNA of 5 µl NASBA reaction was captured on streptavadin coated magnetic dynabeads (Dynal) with the biotynylated oligonucleotide OT 700 (5' Biotin-TGTTAAAAGAGACCHTCAAT-GAGGA 3') as intermediair. The capture hybridization process takes place at 45 °C for 30 minutes in 100 µl hybridization buffer II (5 x SSPE, 0.1% SDS, 0.1% milkpowder, 10 µl/ml denatured salm-sperm DNA; Sambrook et al., 1989). After this step the beads are washed in 2 x SSC, 0.1% BSA using a magnet to retain the beads in the reaction tube or microtiter plate.

Subsequently the RNA was hybridized with Horse Radish Peroxidase (HRP) labelled oligonucleotides specific for the WT^- or Q-RNA sequence between before mentioned PstI and SphI sites, in 100 µl hybridization buffer II for 30 minutes at 45 °C.

Non-hybridized HRP-oligonucleotides are washed away using the same procedure described above. Detection of HRP retained on the beads is accomplished by addition of 100 µl substrate solution (0.45 mM TMB.HCl.H_2O, 0.5 mM CTAB, 7.65 g/l Emdex, 27 mM NaCitrate.2H_2O, 22.1 mM citric acid.H_2O, 2.25 mM urea-peroxid and 5.35 mM 2-chloro-acetamid).

The reaction is stopped at an appropriate time point with 50 µl 250 mM oxalic acid. The amount of substrate conversion from colorless to yellow is determined by measuring the absorbance at 450 nm in an Organon Teknika 510 microplate reader. The A_{450} values for both WT^- and Q-probe are analysed as before (figure 7).

The results in figure 7 show an input of 2.7×10^2 molecules WT-RNA using the formula:

$$\log (\text{conc. WT}) = \frac{\log ([Q] \text{ Sig Q } 50\%) + \log ([Q] \text{ Sig. WT } 50\%)}{2}$$

Table 5

Tube	copies WT^- RNA	copies Q-RNA
1	10^2	-
2	10^2	10^2
3	10^2	10^3
4	10^2	10^4
5	10^2	10^5
6	10^2	10^6

References:

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Claims

1. A method of quantifying a target nucleic acid in a test sample by adding a known number of molecules

of a corresponding nucleic acid comprising a well-defined mutant sequence to said test sample said mutant sequence being discriminatory from the target nucleic acid, subsequently performing a competitive amplification reaction of the nucleic acid, and then quantifying the amplified nucleic acid by differential detection.

2. Method according to claim 1 characterized in that the target nucleic acid is ribonucleic acid.

3. Method according to claim 2 characterized in that the known number of molecules are RNA molecules.

4. Method according to claim 1, 2 or 3 characterized in that the differential detection is performed by using two probe sequences capable of hybridizing with the target nucleic acid and the mutant sequence.

5. Method according to claim 1, 2 or 3 characterized in that the differential detection is performed by using a probe sequence capable of hybridizing with both the target nucleic acid as well the mutant sequence.

6. Method according to claim 1, 2 or 3 characterized in that the differential detection is performed by using a ribozyme capable of cleaving the mutant sequence or target nucleic acid.

7. Test kit for carrying out the method according to claim 1 comprising a well-defined mutant sequence, and appropriate oligonucleotides.

8. The test kit of claim 7 wherein the well-defined mutant sequence is GACAGTGTAGATAGATGACAGTCGCATG.

FIGURE 1

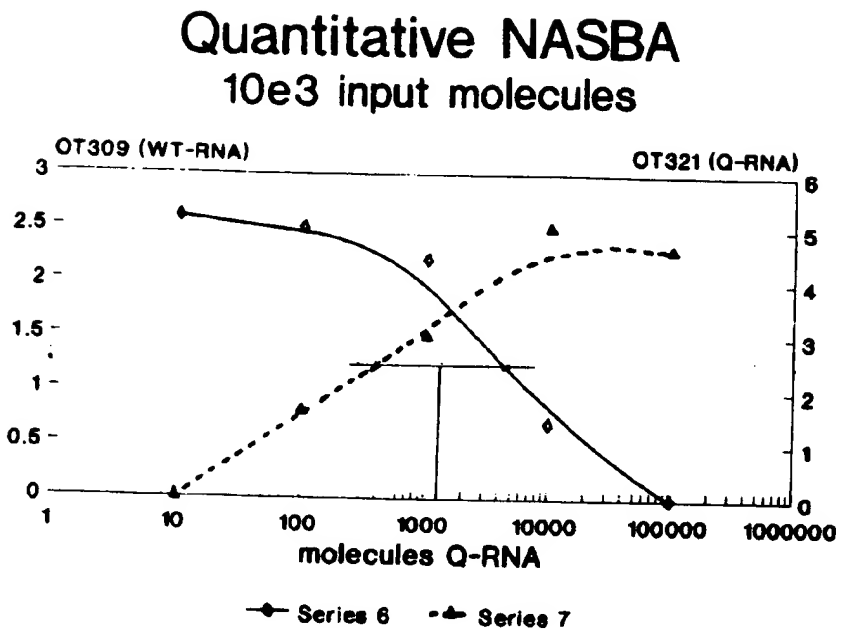


FIGURE 2

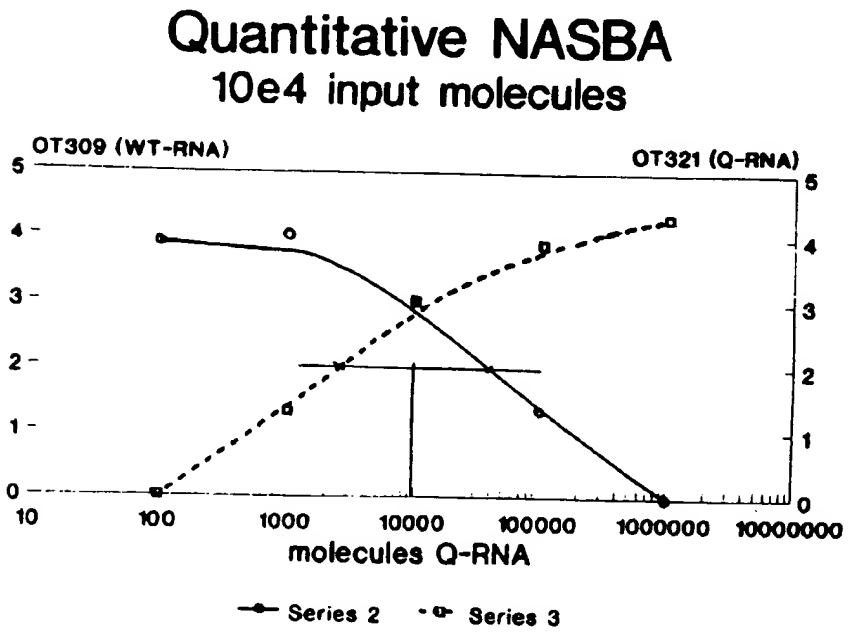


FIGURE 3

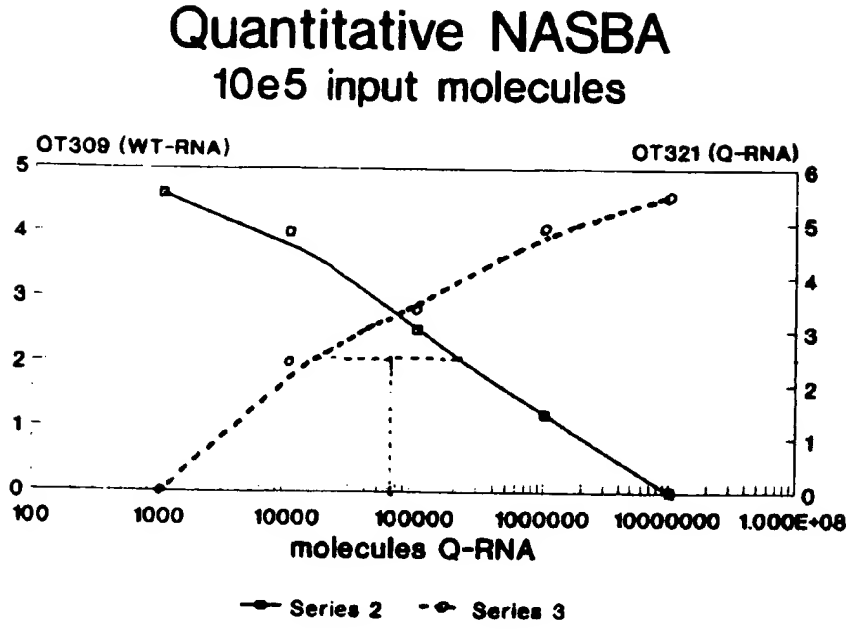


FIGURE 4

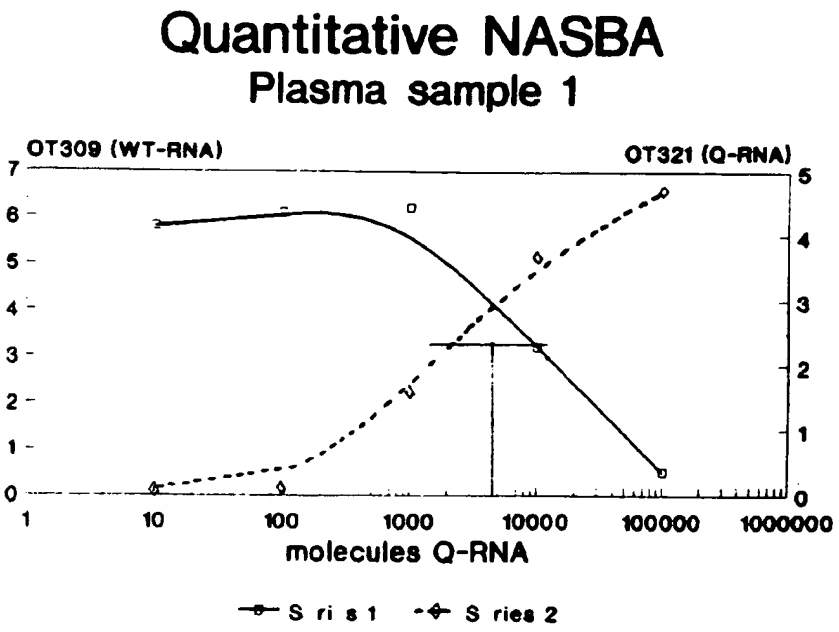


FIGURE 5

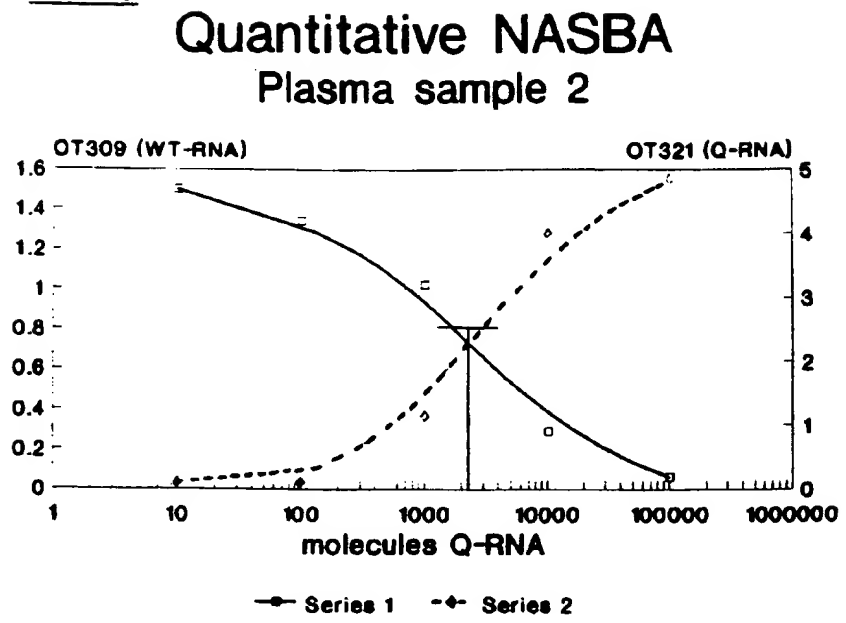
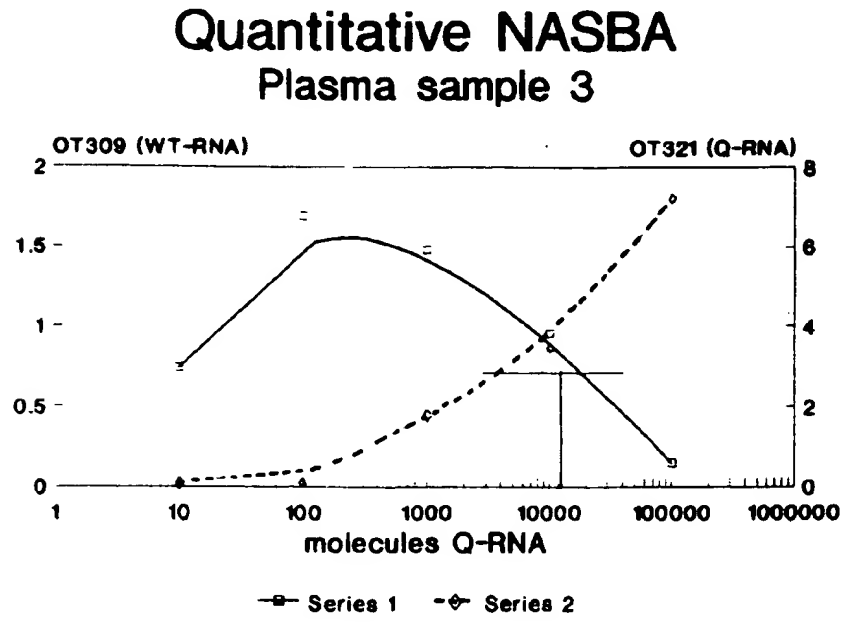


FIGURE 6



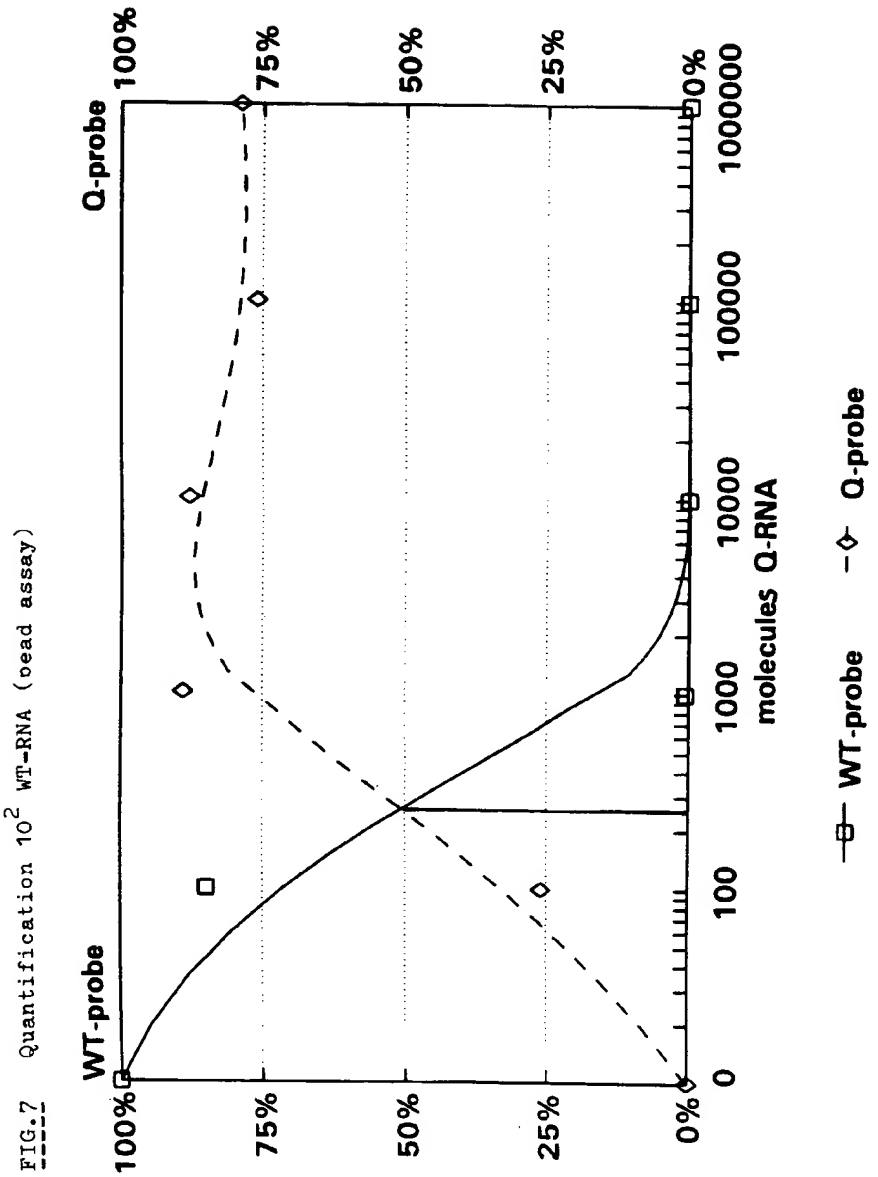


FIG.7 Quantification 10^2 WT-RNA (bead assay)

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EUROPEAN SEARCH REPORT

Application Number

EP 92 20 2238

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	WO-A-9 102 815 (DIAGEN INSTITUT FÜR MOLEKULARBIOLOGISCHE DIAGNOSTIK GMBH) * page 16, line 25 - page 17, line 33; figures 8,9 * ---	1-7	C12Q1/68 C12Q1/70
X	ABST AN MEET AM SOC MICROBIOL vol. 90, 1990, page 114 B. LAMBE ET AL. 'Quantitation of Epstein-Barr Virus (EBV) DNA in clinical specimens utilizing the polymerase chain reaction (PCR)' * abstract * ---	1-2,4	
X	NUCLEIC ACIDS RESEARCH. vol. 17, no. 22, 1989, ARLINGTON, VIRGINIA US pages 9437 - 9446 M. BECKER-ANDRÉ 'Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY)' * the whole document * ---	1,2,6,7 4,5	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C12Q
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, April 1990, WASHINGTON US pages 2725 - 2729 G. GILLILAND ET AL. 'Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction' * the whole document * ---	1,2,6,7 4,5	
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	30 OCTOBER 1992	MOLINA GALAN E.

CATEGORY OF CITED DOCUMENTS

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EP 92 20 2238

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	NATURE. vol. 324, 13 November 1986, LONDON GB pages 163 - 166 R. K. SAIKI ET AL. 'Analysis of enzymatically amplified beta-globin and HLA-DQalpha DNA with allele-specific oligonucleotide probes' * abstract * ---	4,5	
D,A	WO-A-9 102 817 (CETUS CORP.) -----		
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	30 OCTOBER 1992	MOLINA GALAN E.

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& : member of the same patent family, corresponding document

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